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Candidate Reference Method for Determination of Total Bilirubin in Serum: Development and Validation

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This candidate Reference Method for measuring total bilirubin in serum is based on the Jendrassik–Gróf principle (*Clin Chem* **29:** 297–301, 1983). Standard Reference Material no. 916 bilirubin (National Bureau of Standards) is used as the standard. Bilirubin standard solutions may be prepared either in human serum or in 40 g/L albumin solution (human or bovine), because we found the molar absorptivity of the azopigment at 598 nm to be identical in these media. The absorptivities of the unconjugated and conjugated azopigments appear to be identical, but the conjugated azopigment is completely hydrolyzed in the final reaction mixture. Bilirubin added to serum from adults or neonates was quantitatively accounted for. Interference by hemoglobin (up to 2 g/L), ascorbic acid (up to 20 mg/L), or zinc (at physiological concentrations) is negligible. Of the therapeutic drugs we tested, only L-dopa and α-methyldopa interfere. We established normal adult reference values for total bilirubin and examined the intraindividual variation in 19 subjects.

Additional Keyphrases: *azopigments · Jendrassik–Gróf technique · reference interval · sex- and age-related effects · intra-individual variation · neonates · isomers, absorption studies*

In 1974, the Committee on Standards of the American Association for Clinical Chemistry established a study group on bilirubin (BIL), the goal of which was to develop a candidate Reference Method for serum total bilirubin (TBIL).[5]

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[5] Nonstandard abbreviations: SRM, Standard Reference Material (from NBS, the U.S. National Bureau of Standards); HSA, human serum albumin (or solution of it); BSA, bovine serum albumin (or solution of it); HPLC, "high-performance" liquid chromatography; BIL, bilirubin; TBIL, total bilirubin; B_u, unconjugated bilirubin; B_c, conjugated bilirubin; dB_c, bilirubin diglucuronide; mB_c, bilirubin monoglucuronide; and B_δ, δ-bilirubin.

Developed for the Committee on Standards of the American Association for Clinical Chemistry by the Study Group on Bilirubin, with the cooperation of the National Bureau of Standards and the Food and Drug Administration.

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The Jendrassik–Gróf (*1*) principle was chosen as the basis of the method, because extensive evaluations (*2–6*) had demonstrated certain important advantages, among them ease of standardization, high precision, excellent linearity, minimum interference from hemoglobin, rapid color development, constancy of the molar absorptivity of the azopigment in various protein matrices, and adaptability to various instruments for automated chemical analysis. We did not consider methods requiring other kinds of accelerators such as methanol (*7, 8*) and surfactants (*9, 10*). The disadvantages of methanol have been amply documented (*2, 6, 11*), and the experience with surfactants was rather limited.

Four BIL species have been identified in human serum: unconjugated (B_u), monoglucuronide (mB_c), diglucuronide (dB_c), and δ-bilirubin (B_δ). Unlike the others, the BIL in B_δ is firmly bound to protein (*12–15*). "High-performance" liquid chromatography (HPLC) has been used for measuring TBIL in serum after the four individual BIL species were added (*16*). The TBIL values by HPLC correlate well with those of the Jendrassik–Gróf method (*16*). However, the accuracy and precision of HPLC in the measurement of serum TBIL are at present inadequate because (*a*) calibration is performed with B_u with the assumption that the molar absorptivities of the other three bilirubin species are identical to that of B_u, although, in fact, they are not known; (*b*) errors in the measurements of the fractions are cumulative; and (*c*) some BIL may be lost during the complex pretreatment of samples. Thus, we did not consider HPLC to be the method of choice—although future developments may alter this opinion. The proposed method has been tested for transferability (*17*). We now report data on the validation of the method, i.e., linearity, accuracy, molar absorptivity of SRM 916, optimized conditions for measurement of TBIL, interferences, variability in reagents, chemical nature of the alkaline azopigments, and reference values.

Principle

Bilirubin (B_u, mB_c, dB_c, and B_δ) reacts with diazotized sulfanilic acid (diazo reagent) in the presence of caffeine–benzoate–acetate reagent and is converted into azopigments. The reaction occurs at the methylene carbon atom between rings B and C, with the formation of one molecule each of azopigment and hydroxypyrromethene carbinol. A second molecule of azopigment is formed from the reaction between this carbinol and diazo reagent (*18*). Addition of alkaline tartrate shifts the absorption maximum from 530 nm to 598 nm.

Materials and Methods

Equipment

Instruments. We used Cary Model 16 and 210 spectrophotometers (Varian, Inc., Palo Alto, CA 94303) and "Suprasil (QS)" cuvets (specified lightpath, 10 ± 0.01 mm; Hellma Cells, Inc., Jamaica, NY 11424) for absorbance measurements. The photometric accuracy and linearity of the instruments were established and regularly checked by the use of standard glass filters (SRM 930; National Bureau of Standards, Washington, DC 20234). A similar glass filter having an absorbance near 1.6 was calibrated and provided by Dr. R. Mavrodineanu of NBS.

Pipets. We used either Class A volumetric pipets or mechanical pipettors (Micromedic Systems, Inc., Philadelphia, PA 19106).

Materials

Bilirubin. We used "Reference-grade" (Pfanstiehl Laboratories, Inc., Waukegan, IL 60085) bilirubin and SRM 916 bilirubin (NBS).

Human serum albumin (HSA). HSA, L-cystine-treated and de-ionized (*19*), was donated by Hyland Division, Travenol Laboratories, Inc., Costa Mesa, CA 92626.

Bovine serum albumin (BSA). BSA (Cohn Fraction V, lots M45001, M45703, and N50404) was from Metrix Clinical Diagnostics, Division of Armour Pharmaceutical Co., Chicago, IL 60616.

A collection of samples of human and bovine serum albumin (Cohn Fraction V or crystalline) from various manufacturers was provided by NBS. A 60 g/L solution of highly purified HSA, consisting almost entirely of the monomer, was donated by Miles Laboratories, Inc., Elkhart, IN 46515.

Pooled sera from hospitalized individuals were clear, nonhemolyzed, and non-jaundiced, and were used either fresh (24-h old or less) or old (stored frozen for several months).

Reagents and Standards

Use reagent-grade chemicals, when available, and distilled or de-ionized water with a specific resistance (resistivity) of at least $10^8\ \Omega \cdot m$ at 25 °C.

Caffeine reagent. Dissolve 56.0 g of anhydrous sodium acetate, 56.0 g of sodium benzoate, and 1.0 g of disodium EDTA in about 500 mL of water. Add 37.5 g of caffeine, stir until completely dissolved, and dilute to 1 L. Filter if turbid and store at room temperature. This reagent is stable for at least six months.

Alkaline tartrate. Dissolve 75.0 g of NaOH and 320.0 g of potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) in water and dilute to 1 L. Filter if turbid. Stored at room temperature, this reagent is stable for at least six months.

Sodium nitrite, 5.0 g/L. Prepare freshly every two weeks, and store at 4 °C.

Sulfanilic acid, 5.0 g/L. Dissolve the sulfanilic acid in water, add 15.0 mL of concentrated HCl, and dilute to 1 L with water. Store at room temperature. This reagent is stable for at least six months.

Diazo reagent. Just before use, mix 1.0 mL of the sodium nitrite solution with 40 mL of the sulfanilic acid solution.

Tris buffer, 0.1 mol/L, pH 7.3. Dissolve 12.1 g of tris(hydroxymethyl)methylamine (Tris) in 800 mL of water, adjust to pH 7.30 ± 0.01 with HCl, and dilute to 1 L. Store at 4 °C. Discard when there is evidence of microbial or mold growth.

Bovine serum albumin, 40 g/L. Dissolve the albumin in the Tris buffer. If necessary, adjust the pH of the solution to 7.35 ± 0.05 before diluting to final volume with Tris buffer. This solution is used in preparing the stock and working BIL standard solutions. Store it at 4 °C for two to three days, or, for long storage, at −20 °C.

Stock bilirubin standard, 200 mg/L. Use SRM 916 bilirubin or its equivalent for preparing the BIL standard solution, which is done under as little lighting as practicable, both in preparing and in using the BIL standards.

Use a plastic weighing dish (Dispo Weigh Boat, cat. no. B2045-5; American Scientific Products, Div. Am. Hosp. Supply Corp., McGaw Park, IL 60085) to weigh the BIL; *do not use glassine paper.*

Note: The effects of static electricity may make it necessary to take several minutes to obtain a constant weight on the plastic dish from the time it is placed on the balance pan. Therefore, do not weigh the BIL until the weight of the plastic dish becomes constant. Weigh about 40 mg of BIL to the nearest 0.01 mg and transfer it to the bottom of a 100 mL volumetric flask. While keeping the weighing dish at the opening of the flask, use 2.0 mL of dimethyl sulfoxide to quantitatively wash into the flask any BIL that sticks to the dish or the neck of the flask. Swirl the contents of the flask until all of the BIL is finely dispersed; this may take about 1 min. Wash the weighing dish and neck of the flask with 4.0 mL of 0.1 mol/L aqueous Na_2CO_3 solution. Swirl gently until a crystal-clear, red-orange solution is obtained. Dilute the BIL solution to volume with the 40 g/L BSA solution, and mix well by inversion.

Notes: 1. To disperse any foam formed during addition of the BSA solution or after mixing, moisten a wooden applicator stick with caprylic alcohol (remove excess alcohol by touching the stick on filter paper) and move the moistened end over the foam in a circular motion.

2. Failure to dissolve all of the BIL in the dimethyl sulfoxide–sodium carbonate mixture will result in an inaccurate standard. Any undissolved BIL, which remains insoluble after protein is added, can be detected by centrifuging a few milliliters of the final solution; an orange sediment can be seen at the bottom or on the wall of the tube.

Standard blank. Into a 200-mL volumetric flask, add 2.0 mL of dimethyl sulfoxide and 4.0 mL of 0.1 mol/L Na_2CO_3, and dilute to volume with the 40 g/L solution of BSA.

Working bilirubin standards. Dilute the stock bilirubin standard with the standard blank to give BIL concentrations of 5, 10, 15, 20, 50, 100, and 150 mg/L. Dispense these solutions into polypropylene tubes and store at −70 °C. These standards are stable for at least six months (*6*).

Analysis of the Stock Standard BIL Solution

Use Class A volumetric pipets only. Analyze the stock BIL standard and the standard blank in quadruplicate by the procedure described below. Do not run sample (sulfanilic acid) blanks. Measure the absorbance of the solutions as described below (see *Absorbance measurements*). Subtract the average absorbance of the standard blank from the average absorbance of the standard to obtain the corrected absorbance of the standard. Using the corrected absorbance of the standard, calculate the molar absorptivity (ϵ) of BIL. The stock standard BIL solution is acceptable if the ϵ value is between 74 400 and 76 600 $L\ mol^{-1}cm^{-1}$ at 598 nm. If the ϵ value falls outside these limits, prepare a new stock standard.

Notes: 1. BIL standard solutions and standard blanks may also be prepared with 40 g/L HSA or pooled human sera by following the procedure described above. When a serum pool is used, the Tris buffer is omitted.

2. Bilirubin does not absorb light at 598 nm; the absorption is due to the azopigments derived from the diazo reaction. We followed the accepted convention for calculat-

ıg the ϵ value of the azopigment by disregarding the fact ıat 1 mole of BIL yields 2 moles of azopigment.

ɔpectrophotometry

Use a spectrophotometer that meets the specifications escribed in the test for transferability (*17*). An instrument ıaving a bandpass of <2.5 nm is required if molar absorptiities for standard BIL solutions are to be sufficiently ıccurate.

ɔrocedure

Flow-through cuvets may be used instead of regular ıuvets if carryover is eliminated by thorough rinsing be-ween samples. Use class A volumetric pipets. Mechanical ıipettors may be used if the relative SD for repetitive lelivery approaches 0.3%. A procedure for checking such levices has been described (*21*).

Analyze samples, controls, standards, and sample blanks in duplicate. To avoid problems of carryover, the sample blanks should be run first as a group, followed by the test samples. Observe the usual precautions in handling specimens before and during the analysis (until the coupling reaction is completed): work away from windows and with the laboratory lights off.

Note: Do not run sample blanks on the BIL standard solutions. Instead, analyze the standard blank as a test, and subtract its absorbance from those of the standard solutions.

Test samples ("tests"). Use standard solutions containing 20, 50, 100, 150, and 200 mg of BIL per liter.

1. Pipet 4.0 mL of caffeine reagent into a series of 16 × 125 mm glass tubes.
2. Pipet 0.50 mL of sample (serum, standard, etc.), mix, and allow to stand for 10 min.
3. Add 1.0 mL of diazo reagent, mix *immediately,* and let stand for 10 min at room temperature. Add the diazo reagent at timed intervals (e.g., 0.5 or 1 min) and observe the same interval for the addition of alkaline tartrate and for the absorbance measurement.
4. Add 3.0 mL of alkaline tartrate, mix thoroughly, and let stand for 10 min before measuring the absorbance.

Sample blanks. Follow the same procedure and timing schedule as for the tests (steps 1–4 above), but substitute sulfanilic acid for diazo reagent in Step 3.

Reagent blank. Prepare a large volume of reagent blank by mixing 20 mL of caffeine reagent, 2.5 mL of water, 5 mL of diazo reagent, and 15 mL of alkaline tartrate. The reagent blank is needed for checking the photometric drift of the spectrophotometer.

Absorbance measurements. Use the same cuvet for all measurements. Keep the cuvet in the same orientation with respect to the light beam throughout the run.

1. Set the spectrophotometer at zero absorbance (0.000 *A*) at 598 nm with *air* in both the sample and reference beams (i.e., with no cuvets in the cuvet holder).
2. Fill the cuvet with reagent blank, wipe off fingerprints with lint-free tissue, and measure the absorbance (A_1) vs air in the reference beam. If a flow-through cuvet is used, fill the cuvet with reagent blank and set the absorbance at zero; A_1 in this case will be 0.000 *A*.
3. In the properly timed sequence, read all sample blanks (A_2) and tests (A_3). Rinse the cuvet twice with small aliquots of each solution to be read.
4. Check the photometric drift of the spectrophotometer after every 10 samples as follows: Rinse the cuvet with 3 1-mL portions of the reagent blank, then fill the cuvet with the same solution and measure the absorbance (A_1). In each case, the reading should lie within ± 0.002 *A* of the previous reading. If the difference is greater, repeat the rinsing and reading, after wiping off any fingerprints on the cuvet. If the drift still exceeds ± 0.002 *A*, set the spectrophotometer at 0.000 *A*, with air in both beams.

Notes: 1. If drift presents an irremediable problem, do not perform the analysis until the instrument is repaired.

2. The reading A_1 is used only for checking the photometric drift, not for correcting A_2 or A_3. The reagent blank is used because it has the same viscosity as the tests and the sample blanks.
3. A procedure (optional) for making absorbance measurements with the cuvet in place (without removing it from the cuvet holder) has been described elsewhere (*22*).
4. The difference between A_2 and A_1 ($A_2 - A_1$) is the net absorbance of the sample blank.

Calculations and Acceptability of Data

Subtract the average absorbance of the standard blank from the absorbance of each standard to obtain the corrected readings of the standards. Subtract the average absorbance of each sample blank (A_2) from each duplicate value of the corresponding test (A_3) to obtain the corrected readings of the tests.

First condition. The differences between readings for duplicate serum specimens, controls, standard solutions, standard blank, and sample blanks should not exceed 0.005 *A* or 1% of the absorbance value, whichever is greater. If differences are greater, re-analyze those specimens.

If the first condition is met, evaluate the linearity of the standard curve by linear regression analysis of the data (x = concentration of the standard, y = absorbance).

Second condition. The linearity is acceptable if the intercept is 0.000 ± 0.005, r^2 (Pearson's coefficient) exceeds 0.999, and the concentrations of the standards calculated from the regression equation are within ± 2% of the nominal values or 2 mg/L, whichever is greater. If volumetric pipets are used, the slope for the BIL standards should be between 0.00749 and 0.00771.

If both of the above conditions are met, the run is acceptable, and the total bilirubin concentration in the serum specimens is calculated from the equation

$$x = (y - a)/b$$

where: x = concentration in the sample, in mg/L
y = corrected absorbance of specimen
b = slope
a = intercept.

Optional Procedure for Specimens with Low BIL Concentrations (5–20 mg/L)

The following optional procedure may be used for analyzing serum samples having BIL concentrations near the physiological range. This procedure, threefold as sensitive as the procedure described above, provides increased accuracy and precision at low BIL concentrations. Although the optional procedure has not been tested for transferability, our experience (at the Medical College of Wisconsin) indicates that it is as valid as the proposed method.

Use standard solutions containing 5, 10, 15, and 20 mg/L BIL. Follow the procedure described above, but use the following volumes: caffeine reagent, 1.0 mL; sample, 0.50 mL; diazo reagent, 0.50 mL; alkaline tartrate, 1.0 mL.

The measurement of absorbance, calculations, and criteria for acceptability of data are the same as for the proposed method.

Validation of the Method

Time Required for Completion of the Diazo Reaction

In the presence of caffeine reagent, B_u reacts completely with the diazo reagent in 1 min (*23*). Lo and Wu (*24*) have shown that the reaction of dB_c is also complete with the

proposed method. We analyzed a human serum pool enriched with a mixture of dB_c and mB_c, isolated from human bile (*25*), and found no increase in color when the coupling reaction was prolonged beyond 10 min. The percentage composition of the enriched serum pool, which was established with the use of HPLC by Dr. William Fellows of the Eastman Kodak Company, was: B_δ, 7%; mB_c, 49%; dB_c, 38%, B_u, 6%.

Although TBIL values (for 357 pathological sera) by the Jendrassik–Gróf procedure were in good agreement with those obtained by HPLC (*16*), it is not entirely certain whether all of the B_δ in serum reacts quantitatively in the proposed method. In our laboratory (Medical College of Wisconsin) we have shown that TBIL values for jaundiced sera from adults remain essentially unchanged when we increase the coupling time from 10 to 20 min (Table 1). In another experiment we analyzed jaundiced samples from adults with use of increased concentrations of diazotized sulfanilic acid in the diazo reagent, keeping the coupling time to 10 min. The results (Table 2) show that TBIL values in these patients' sera are higher by no more than 4% when the diazo concentration is increased fourfold, while the B_u absorbance is decreased by 1%.

Stability of Bilirubin in the Caffeine Reagent

Reportedly, BIL is very unstable in the caffeine reagent (*5*), so we delayed the addition of the diazo reagent to the caffeine–sample mixture for up to 30 min while keeping the solution in the dark. We found that BIL is stable during the 30-min period, but reproducible absorbance readings are obtained only after the sample has been in contact with the caffeine reagent for at least 5 min before the diazo reagent is added (data not shown). We recommend that the diazo reagent be added 10 min after addition of sample to caffeine.

Note: For brevity, some of the data are not shown, but will be made available upon request to one of the authors (B. T. D.).

Table 1. Effect of Coupling Time on TBIL Values (mg/L) Obtained for Jaundiced Sera from Adults

Sample no.	Time, min		
	10	15	20
1	53.9	54.4	54.7
2	67.6	67.8	67.8
3	62.1	62.4	62.4
4	22.6	22.8	23.0
5	37.9	38.0	38.0
6	74.9	75.8	75.2
7	83.6	84.2	84.6
8	100.5	101.3	101.5
9	77.6	77.9	78.1

Table 2. Effect of Diazo Reagent Concentration on Values Obtained for TBIL in Jaundiced Sera from Adults

	Diazo concn, mmol/L[a]				
	1.8[b]	3.5	7.0	10.5	14.0
	Absorbance				
B_u in serum (50.8 mg/L)	0.301	0.298	0.298	—	—
B_u in serum (203.1 mg/L)	1.196	1.188	1.181	1.162	1.129
	TBIL, mg/L				
Serum no. 1	72.4	73.0	75.1	73.5	71.7
Serum no. 2	118.8	121.0	122.3	119.8	116.9
Serum no. 3	235.4	238.8	241.1	238.3	229.3

[a] $NaNO_2$ concentration in the diazo reagent.
[b] Concentration of diazo reagent in the candidate Reference Method.

Linearity

We evaluated the absorbance–concentration relationship with use of B_u solutions in pooled sera, or solutions in BSA or HSA, and with jaundiced specimens having high BIL concentrations, from adults. Stock B_u solutions were diluted with the corresponding standard blank solutions, patients' sera with 9 g/L NaCl solution. Figures 1–3 show the linearity for B_u in normal human serum and in patients' sera. Results were identical for B_u in HSA and BSA (data not shown).

Molar Absorptivity of Bilirubin

At the Medical College of Wisconsin, SRM 916 has been used during the past 13 years for preparing standard

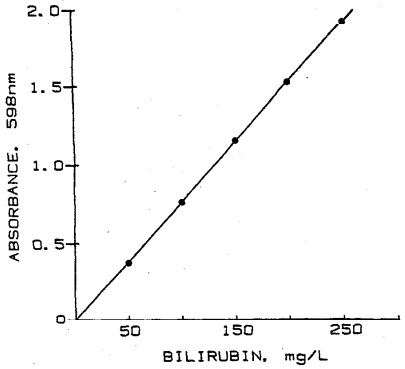

Fig. 1. Absorbance–concentration relationship for B_u in human serum with the candidate Reference Method
$y_{abs} = 0.769 \times 10^{-2} (\pm 4 \times 10^{-5})\ x_{concn} - 0.003\ (\pm 0.006)$; $r^2 = 1.0$; $S_{y \cdot x} = 0.006$. In Figs. 1–3, the first ± expression is SE of slope, second is SE of intercept.

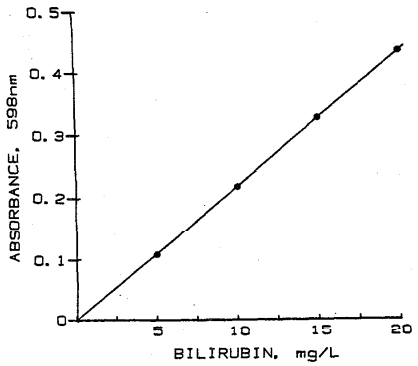

Fig. 2. Absorbance–concentration relationship for B_u in human serum with the proposed optional method
$y_{abs} = 2.17 \times 10^{-2} (\pm 6 \times 10^{-5})\ x_{concn} - 0.001\ (\pm 0.001)$; $r^2 = 1.0$; $S_{y \cdot x} = 0.0007$

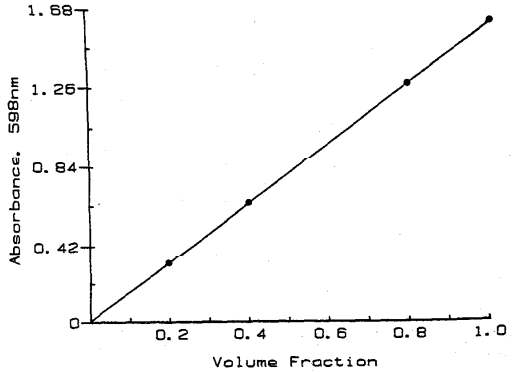

Fig. 3. Absorbance–concentration relationship for icteric serum pool; the BIL concn in the undiluted pool was 269 mg/L
$y_{abs} = 1.594\ (\pm 0.014)\ x_{vol.fr.} + 0.006\ (\pm 0.010)$; $r^2 = 1.0$; $S_{y \cdot x} = 0.009$

solutions in HSA, BSA, or human serum. The values obtained for the molar absorptivity at 598 nm of 24 freshly prepared BIL solutions were:

$$\bar{x} = 75\,500 \text{ L mol}^{-1}\text{cm}^{-1};\ SD = 550$$

Actual range: 74 600 to 76 400 L $mol^{-1}cm^{-1}$

The limits ($\bar{x} \pm 2$ SD) thus appear to be from 74 400 to 76 600 L $mol^{-1}cm^{-1}$. Novros et al. (*10*) and Vink et al. (*26*) reported values of 74 800 and 75 100, respectively.

On one occasion, we prepared and analyzed, simultaneously, solutions of SRM 916 and of a BIL preparation consisting of the IX-α isomer only (from BDH Chemicals Ltd., Poole, England; purified at NBS by the same procedure as used for SRM 916). The molar absorptivities for the SRM and the IX-α bilirubin were 75 100 and 74 900 L $mol^{-1}cm^{-1}$, respectively, a difference well within the experimental error of the measurement.

Color Stability

With BIL in HSA or BSA, the color of the azopigment is stable for at least 30 min. With bilirubin in serum, there is a small increase in absorbance with time in both tests and sample blanks, caused by a gelatinous precipitate, slowly formed in both tests and sample blanks. To evaluate the magnitude of the problem, we analyzed sera with low and high concentrations of TBIL and measured the absorbance of both tests and blanks 5, 10, 20, and 30 min after addition of alkaline tartrate. Identical results for TBIL were obtained when sample blanks and tests were read at the same time, for the absorbances of both increased to the same extent. For most of the serum samples, the increase in absorbance with time was <0.003 *A*; in two samples (both lipemic), the increase in absorbance was 0.020 *A*. We recommend that absorbance measurements on both tests and blanks be made at the same time (see *Procedure*).

Variability in Reagents

Throughout this study we purchased reagents from several sources. The narrow range of molar absorptivity values for the SRM 916 obtained with use of reagents from various sources indicates that the imposition of strict specifications on the purity of reagents required for the proposed method is not warranted. However, owing to the possibility of reagent contamination by certain heavy metals (see *Interferences*) and because we found that the ϵ value of the azopigment depended somewhat on the source of sodium tartrate used, we examined several lots of sodium tartrate, sodium potassium tartrate, and NaOH.

Alkaline tartrate solutions were prepared with (*a*) a single lot of NaOH and tartrate (Na or K Na) from different lots and suppliers; and (*b*) a single lot of K Na tartrate and NaOH from different sources and lots. A B_u solution in BSA was analyzed with each set of alkaline tartrate solutions in a single run. The data in Table 3 show that the sources of K Na tartrate and NaOH had a negligible effect on the absorbance of the azopigment (see Table 5). Indeed, the SDs for K Na tartrate and NaOH are similar to the estimates of the within-run precision of the method. However, the use of Na tartrate was abandoned because of the larger variation in the absorbance values.

Table 3. Absorbance Values for Azopigment Obtained with Alkaline Tartrate Reagents Prepared with Na Tartrate, K Na Tartrate, and NaOH from Various Sources and Lot Numbers

	Absorbance[a]	
	Observed range	SD
Na tartrate (4)[b]–NaOH (1)	0.716–0.728	0.005
K Na tartrate (8) –NaOH (1)	0.715–0.718	0.0006
K Na tartrate (1) –NaOH (12)	0.674–0.680	0.0021

[a] Three different B_u solutions were used in these experiments.

[b] Numbers in parentheses are the numbers of lots used for the preparation of each alkaline tartrate reagent.

Concentration of Reagents

To decrease the undesirably high viscosity of the caffeine reagent used in our original procedure (*6*), in all of the present experiments we used a caffeine reagent diluted to 75% of its original strength. To test the effect of the caffeine reagent strength on the reactivity of B_u and other BIL species, we analyzed with use of caffeine reagent of various concentrations: (*a*) B_u solutions in HSA, BSA, or pooled sera; (*b*) two pools from jaundiced sera from adults; and (*c*) a homemade control containing B_c isolated from human bile. The data in Table 4 confirm previous findings on the reactivity of B_u (*6*) and demonstrate that absorbance values for the two pooled sera and the B_c preparation remain constant when the concentration of the caffeine reagent is widely varied.

The molar ratio of sulfanilic acid to $NaNO_2$ is about 16:1. The large excess of sulfanilic acid is necessary for obtaining maximum velocity in the second step of the coupling reaction (*23, 25*). When the molar ratio is decreased to 3:1 (1 g of sulfanilic acid per liter), the ϵ value of the azopigment at 598 nm is about 4% lower (*26*), probably because the coupling reaction does not go to completion.

The amount of $NaNO_2$ in the diazo reagent (1.77 μmol in 1 mL) is theoretically enough to react with 0.88 μmol of BIL, which under the conditions of the assay corresponds to a BIL concentration of 1 g/L in serum. We have found that, with this amount of diazo reagent, at least 0.28 μmol of B_u reacts quantitatively, and that a linear absorbance concentration relationship is obtained in the analysis of jaundiced serum containing 269 mg of TBIL per liter (Figure 3).

All of the diazo reagent is not, however, available for reacting with BIL, for some of it is consumed in side reactions. We have found that the diazo reagent reacts with acetate and benzoate, ingredients of the caffeine reagent, forming with both a yellow color that exhibits a strong absorption peak at 410 nm. Under the assay conditions the absorbance values for acetate and benzoate are 3.6 *A* and 2.9 *A*, respectively. Some of the diazo reagent is expected to react also with the free carboxyl groups of the serum proteins.

Table 4. Effect of the Concentration of Caffeine Reagent on the Completion of the Diazo Reaction

	Absorbance					
Reagent concn, % of full strength[a]	B_u			Adult sera		B_c
	In HSA	In BSA	In serum	Pool 1[b]	Pool 2[c]	In serum
100	1.522	1.527	1.497	1.076	0.567	0.494
90	1.522	1.517	1.493	1.070	0.567	0.498
80	1.524	1.521	1.495	1.073	0.568	0.499
70	1.518	1.522	1.496	1.073	0.567	0.497
60	1.519	1.520	1.501	1.068	0.562	0.498
50	1.523	1.530	1.500	1.076	0.564	0.498
40	1.524	1.525	1.481	1.067	0.563	0.501
30	1.531	1.532	1.253	1.070	0.561	0.500
20	1.512	1.495	0.918	1.065	0.560	0.505
10	1.308	1.184	0.690	0.974	0.560	0.502

[a] The full-strength reagent contains 50 g of caffeine, 75 g of sodium benzoate, and 75 g of sodium acetate per liter.

[b] Total bilirubin, 140 mg/L; direct, 95 mg/L.

[c] Total bilirubin, 75 mg/L; direct, 56 mg/L.

Effect of Protein Matrix on the Absorptivity of B_u and Its Alkaline Azopigment

The following experiment was performed, on three occasions, to evaluate the effect of the protein matrix on the absorptivity of B_u and its azopigment. The protein matrices examined were: eight BSA preparations (Cohn Fraction V or crystalline), five HSA preparations (one of them being an HSA monomer provided by Miles Laboratories, Inc.), and 33 serum samples from hospitalized individuals.

We dissolved about 7 mg of B_u in a mixture of 0.5 mL of dimethyl sulfoxide and 1 mL of 0.1 mol/L Na_2CO_3, and diluted the solution to 10 mL with water. We added 0.1-mL aliquots of this solution to 0.5 mL of each protein ("tests") with a MicroStat pipet (Micromedic Systems, Inc.). "Blanks" were prepared in the same manner, except that water was substituted for the bilirubin solution. "Tests" and "blanks" were set in duplicate. All samples were analyzed by the proposed method 5 min after the bilirubin was added to protein. The absorbance values of the "blanks" (analyzed as "tests," i.e., with diazo reagent) were subtracted from those of the "tests" to correct for endogenous bilirubin or other pigments present in the protein matrices. Results from these experiments demonstrate that the absorptivity of the azopigment is remarkably constant in all of the tested matrices (Table 5); CVs vary from 0.2% to 0.3%.

Bilirubin added to the HSA monomer gave an absorbance value at 598 nm that was about 3.6% higher than the mean value obtained in the other protein matrices. An explanation for this puzzling finding was provided when this HSA solution was found by analysis to contain 92 μmol of zinc per liter (see *Interferences*).

On one occasion, "tests" and "blanks" were diluted with Tris buffer (0.1 mol/L, pH 8.5), and the absorbance of the "tests" was measured at their absorption maxima and corrected with the corresponding blanks. The absorptivity of B_u in Tris buffer is matrix-dependent (Table 6) and, even in serum, it shows a variation about eightfold that of the azopigment.

Interferences

Metal ions. Heirwegh and Van Roy (*27*) have shown that certain metal ions, added to azopigment solutions, induce changes in both the absorption spectrum and the ε values for the azopigment. However, the high metal concentration (0.9 mmol/L in the reaction mixture) they use in their experiments is unlikely to be found in reagents used in the BIL assay. We re-examined the effect of metal ions and found that Mg, Ca, Cd, Ni, Al, Mn(II), Hg(II), Fe(II), and Fe(III), each alone in a concentration of 7 mg/L (≤0.3 mmol/L) in the final reaction mixture, had no effect on the ε value of the azopigment at 598 nm, whereas Fe(II) at concentrations of 0.6 and 3 mmol/L caused a decrease in the ε value at 598 nm of 34 and 93%, respectively.

Zinc, even at very low concentrations, causes a substantial increase in the value for the azopigment at 598 nm, while Pb and Cu, at much higher concentrations than Zn, cause a slight decrease (Table 7). In these experiments the metal salts were added to alkaline tartrate. However, addition of Zn to the serum or caffeine reagent produced the same effect as its addition to alkaline tartrate. We analyzed 40 serum samples from adults, having TBIL concentrations ranging from 7 to 320 mg/L, using alkaline tartrate supplemented with 0.3 mmol of Zn per liter (0.1 mmol/L in the final reaction mixture), and without added Zn. Mean TBIL values with and without Zn supplementation were 93 and 95 mg/L, respectively; the largest difference between samples was 5 mg/L.

Because the Zn content in serum is variable and it may be variable in the reagents also, we considered supplementing one of the reagents with a large excess of Zn. However, we abandoned this approach because the constancy of the ε value for the SRM 916, and the absence of large variation in the absorptivity of the azopigment when we used K Na tartrate and NaOH from different sources, indicated that Zn contamination in the reagents was not a significant problem. Lead and copper cause a decrease in the absorbance of the azopigment, but only at concentrations unlikely to be encountered in analytical-grade reagents. Cadmium has no effect on the absorptivity of the azopigment at 598 nm; its

Table 5. Effect of Protein Matrix on the Absorbance of the Alkaline Azopigment at 598 nm

	Absorbance		
B_u in HSA or BSA	Experiment 1	Experiment 2	Experiment 3
n	12[a]	13[b]	—
$\bar{x}$	1.0597	1.0876	
SD of duplicates	0.0012	0.0014	
SD among samples	0.0016	0.0024	
Total SD	0.0020	0.0028	
Total CV, %	0.2	0.3	
B_u in patients' sera	Experiment 1	Experiment 2	Experiment 3
n	11	12	10
$\bar{x}$	1.0619	1.0877	1.2381
SD of duplicates	0.0013	0.0010	0.0003
SD among samples	0.0029	0.0028	0.0031
Total SD	0.0032	0.0030	0.0031
Total CV, %	0.3	0.3	0.25

The amount of B_u added to the samples differed in each experiment, but it was the same within an experiment.

[a] Three HSA and nine BSA preparations were used.

[b] Four HSA and nine BSA preparations were used.

Table 6. Effect of Protein Matrix on the Absorbance of Bilirubin (B_u) in 0.1 mol/L Tris Buffer, pH 8.5

	Absorbance		
	B_u in BSA	B_u in HSA	B_u in sera
n	9	5	12
$\bar{x}$	0.6222	0.6308	0.6192
SD of duplicates	0.0016	0.0022	0.0025
SD among samples	0.0302	0.0127	0.0132
Total SD	0.0302	0.0129	0.0134
Total CV, %	3.7	2.0	2.2
λ_{max}	470–474 nm	461–464 nm	460–464 nm

The same amount of B_u was added to all samples.

Table 7. Effects of Zinc, Lead, and Copper on the Absorbance (*A*) of the Alkaline Azopigment[a] at 598 nm

Zinc		Copper		Lead	
μmol/L[b]	*A*	μmol/L	*A*	μmol/L	*A*
0	0.781	0	0.772	0	0.772
0.7	0.785	15	0.769	10	0.769
1.5	0.788	30	0.766	20	0.763
3	0.795	45	0.760	50	0.746
6	0.808	60	0.758	100	0.728
12	0.832				
120	0.899				
600	0.907				

[a] The source of the azopigment was B_u added to human serum pool. Salts used: $ZnSO_4$; $CuSO_4$; $Pb(CH_3COO)_2$.

[b] Concentration in the final reaction mixture.

effect on the absorptivity, which is as large as that of Zn, occurs at 620 nm because of the shift in the absorption maximum.

The EDTA in the caffeine reagent apparently fails to chelate Zn at the high pH (13.3) of the final reaction mixture. Increasing the EDTA concentration twofold did not abolish the effect of Zn.

Hemoglobin. Known amounts of a hemoglobin solution, prepared according to Sunderman (*28*), were added to two solutions of BIL in human serum. The original BIL solutions and those containing the added hemoglobin were analyzed (*a*) by the proposed method and (*b*) by adding 0.1 mL of a 40 g/L aqueous solution of ascorbic acid before the addition of alkaline tartrate. As shown in Table 8, hemoglobin up to 3 g/L caused no interference when the ascorbic acid was used, but at 10 g/L the hemoglobin suppressed the BIL value by 5 mg/L. In the absence of ascorbic acid, no suppression of BIL values is seen up to 2 g of hemoglobin per liter.

It should be pointed out that, at high hemoglobin concentrations and in the absence of ascorbic acid, the absorbance of the azopigment gradually decreases with time. Added ascorbic acid stabilizes the absorbance, even at a hemoglobin concentration of 10 g/L.

Ascorbic acid. Because ascorbic acid destroys the diazo reagent (*29*), we evaluated its effect on the measurement of TBIL by adding known amounts of ascorbic acid to BIL solutions in serum before analysis. At physiological concentrations, 2–20 mg/L, ascorbic acid suppresses TBIL by 0.6 to 1% (Table 8), a negligible interference.

Lipemia. It is difficult to assess the effect of turbidity in spectrophotometric analysis. Because both the test mixture and the sample blank are of similar chemical composition, light scattering should be of the same magnitude in both solutions if their absorbances are measured at the same time (see *Procedures*). We added a fat emulsion (Intralipid 20%; Cutter Medical, Berkeley, CA 94710) to a bilirubin solution in serum to increase the serum triglyceride content by 4.7 g/L, and the absorbance of the sample blank by 0.40 *A* (about 50 times higher than that seen with clear sera); the TBIL values with and without Intralipid were 94.1 and 96.8 mg/L, respectively.

Other substances. Few compounds have been reported to interfere in the measurement of bilirubin (*30*). We found no interference from the following compounds added to BIL solutions in serum at the concentrations shown in parentheses (mg/L): aminophylline (40), theophylline (40), phenobarbital (80), diazepam (160), ampicillin (120), tobramycin (19), gentamicin (18), tyrosine (200), histidine (200), and a mixture (1250) of amino acids (Travasol™; Travenol Laboratories, Inc., Deerfield, IL 60015). We confirmed the findings of Singh et al. (*31*) that L-dopa and α-methyldopa interfere with the measurement of BIL. In our experiments, concentrations of 85 mg of L-dopa and 65 mg of α-methyldopa per liter of serum caused increases in TBIL of 15 and 6 mg/L, respectively. Indican has previously been shown to not interfere with the Jendrassik–Gróf method (*32*).

Table 8. Interference by Hemoglobin and Ascorbic Acid in the Proposed Total Serum Bilirubin Method

	Bilirubin, mg/L			
	With ascorbic acid		Without ascorbic acid	
Interferent	Pool 1	Pool 2	Pool 1	Pool 2
Hemoglobin, g/L				
0	7.5	100.0	7.4	100.0
0.5	7.3	100.3	7.1	100.0
1.0	7.6	100.1	6.9	99.9
2.0	7.6	100.1	7.3	98.8
2.5	—	99.9	—	98.3
3.0	7.6	99.8	7.1	96.6
10.0	—	95.2	—	81.3
Ascorbic acid, mg/L				
0	20.0	100.0		
5	20.0	100.0		
10	19.8	99.9		
15	19.7	99.6		
20	19.8	99.4		
50	19.4	98.6		
100	19.2	97.4		

Hemoglobin and ascorbic acid were added to B_u solutions in human serum.

Chemical Nature of the Alkaline Azopigments

The demonstrated accuracy of the Jendrassik–Gróf method in the measurement of dB_c (*24*) does not necessarily imply that the absorptivities of the conjugated and unconjugated azopigments are identical. The accurate measurement of dB_c (as well as mB_c) could also be explained by the complete de-esterification of the azopigment in the alkaline medium of the final reaction mixture.

We tried to clarify this uncertainty by following the rate of hydrolysis of the B_c azopigment while simultaneously monitoring the absorbance of the reaction mixture. We analyzed a mixture of mB_c and dB_c in serum and a solution of B_u in BSA as follows: (*a*) we used the diazotized 2,4-dichloroaniline (diluted with water instead of methanol) (*33*) instead of diazotized sulfanilic acid because 2,4-dichloroaniline-produced azopigments are soluble in organic solvents; (*b*) we prepared a large volume of azopigment by adding 1 volume of sample to 4 volumes of caffeine reagent, followed by 2 volumes of diazo reagent and, after 10 min, 0.2 volume of 50 g/L ascorbic acid in water, to destroy excess diazo. The rate of hydrolysis was evaluated by mixing in a series of tubes 7-mL portions of the reaction mixture with 0.5 mL of alkaline tartrate, and adding to each tube at timed intervals 2 mL of 1 mol/L HCl. The solution in each tube was extracted twice with 5 mL of 2-pentanone/butyl acetate (17/3 by vol) (*34*). The extracts, containing both the esterified and B_u azopigments, were evaporated to dryness in a stream of nitrogen, and the residues were redissolved in 0.3 mL of butyl acetate and spotted on precoated glass thin-layer chromatographic plates (Silica Gel 60; Scientific Products, McGaw Park, IL 60085). We developed the chromatograms with $CHCl_3/CH_3OH/H_2O$ (65/25/3 by vol) (*34*) and estimated the relative amounts of the separated B_u and B_c azopigments by scanning with an area-integrating densitometer (Quick Scan; Helena Laboratories, Beaumont, TX 77704). The precentage of B_u originally present in the reaction mixture was estimated by treating in the same manner another 7-mL portion of the reaction mixture, except that the alkaline tartrate was omitted.

A mixture of 7 mL of azopigment and 0.5 mL of alkaline tartrate was used to monitor the absorbance at 590 nm, which did not change during the 30-min experiment. The almost complete hydrolysis of the B_c azopigment (Table 9) indicates that in the final reaction mixture the only azopigment present is that corresponding to B_u. Furthermore, the absorbance would not have been constant during hydrolysis if the absorptivities of the two azopigments were not identical.

Because with 3 mL of alkaline tartrate the hydrolysis is completed in 1 min, we had to use 0.5 mL in order to slow down the rate of hydrolysis and demonstrate that there is no change in absorbance during the de-esterification of the conjugated azopigment.

Table 9. Absorbance Change during Hydrolysis of the 2,4-Dichloroaniline B_c Azopigment by Alkaline Tartrate

Time, min	Absorbance, 590 nm	B_u azopigment, % of total
0	—	30
1	0.600	50
5	0.598	66
10	0.599	71
30	0.599	98
60	0.598	—

Normal Reference Intervals

The reference intervals for serum TBIL reported hitherto have been established with routine methods having poor sensitivity in the physiological range, and with use of calibrators of questionable accuracy.

We obtained data for establishing normal reference values for serum TBIL, using a procedure with good sensitivity in the range of 0 to 20 mg/L and with calibrators prepared from SRM 916.

Blood specimens from 165 medical students and 83 laboratory personnel were collected without anticoagulant; all subjects had fasted for 10–12 h before samples were collected. The specimens were allowed to clot for 1 h, and then centrifuged (1500 × *g*, 15 min). The sera were kept stoppered, in the dark, and were analyzed by the proposed optional procedure on the same day they were collected.

Seven BIL standard solutions (containing 2, 4, 6, 8, 10, 15, and 20 mg/L) and two control sera were included in each run. All samples were analyzed in duplicate. Figure 4 shows the frequency distribution of the TBIL values. Because bilirubin values >12 mg/L are most likely due to Gilbert's syndrome (consistent unconjugated hyperbilirubinemia in the absence of liver disease), which is thought to have a prevalence of about 4 to 5% in the general population (*35*), we excluded 5% of the high BIL values, from both the male and female populations, before statistical treatment of the data. Reference intervals for TBIL, established from the 2.5 and 97.5 percentiles, are shown in Table 10. The obvious difference between mean TBIL values for males and females was confirmed by the Mann–Whitney test (*36*).

Although it has been amply documented (*37–39*) that mean values and ranges for TBIL are dependent upon sex and age, the upper limits of the ranges are so close that, in view of the analytical imprecision of routine methods, it is doubtful that a clear distinction could be made between males and females or among age groups. The considerably higher upper limit for male subjects in the age group of 19 to 36 years reported by Rosenthal et al. (*38*) is difficult to explain; perhaps they did not exclude data from subjects with Gilbert's syndrome. Mean values for TBIL obtained in this study are slightly higher than those reported by other investigators (*37, 38*); our higher values are most likely ascribable to the fasting state of the blood donors (*35*).

We examined the intra-individual (personal) variation in TBIL concentration, with 19 medical students as subjects. Sera were collected weekly for five to 10 weeks and analyzed for TBIL. Figure 5 depicts the lowest and highest TBIL values observed for these subjects. In view of the small SDs of the method, 0.15 and 0.3 mg/L (Table 10), the personal variation in TBIL values (CV ranges from 17 to 42%) appears to be mostly biological, rather than analytical, in origin and it is almost as high as the interindividual variation. Thus, measurement of TBIL cannot contribute much to the personal "blood profile" (*40*). The large fluctuation of TBIL in subject no. 8 (with Gilbert's syndrome) may be due to variations in caloric intake (*41*).

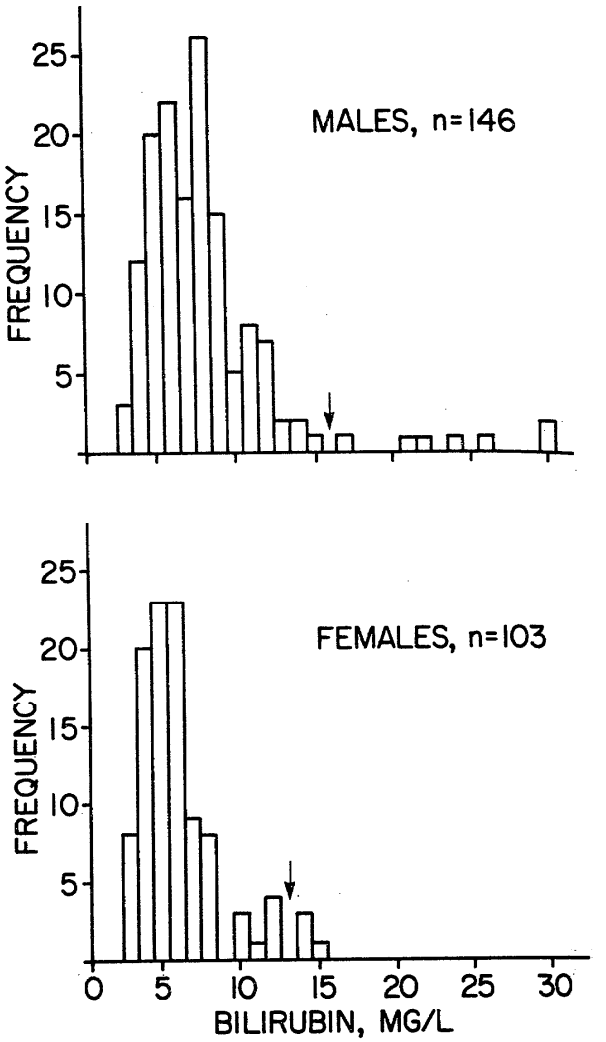

Fig. 4. Frequency distribution of serum total bilirubin values for apparently healthy individuals

146 males and 103 females. Bilirubin values to the right of the *arrows* were not used for establishing the reference interval

Table 10. Normal Reference Values for Serum Total Bilirubin, mg/L

Subjects[a]	$\bar{x}$	2.5 percentile	97.5 percentile	SD
139 ♂	7.0	2.0	12.1	2.5
99 ♀	5.3	2.2	11.2	2.1
Control 1 (n = 24)	5.0	—	—	0.15
Control 2 (n = 13)	12.6	—	—	0.30
Reference *39*				
♂	4[b]	1	11	—
♀	3[b]	1	9	—

[a] 5% of the highest bilirubin values were excluded from the statistical analysis of the data (seven males and four females).

[b] Median.

Analytical Recovery from Serum from Neonates

In view of the importance of BIL determinations in neonates, we checked for the presence of inhibitors of the diazo reaction in such sera. The same amount of B_u, equivalent to 140 mg/L, was added to 14 sera as described under *Effect of protein matrix on the absorptivity of B_u*, etc. Recovery ranged from 98% to 101%, with a mean of 99.5%.

Proposed Optional Procedure

The interferences from hemoglobin and ascorbic acid in this procedure were similar to those observed with the

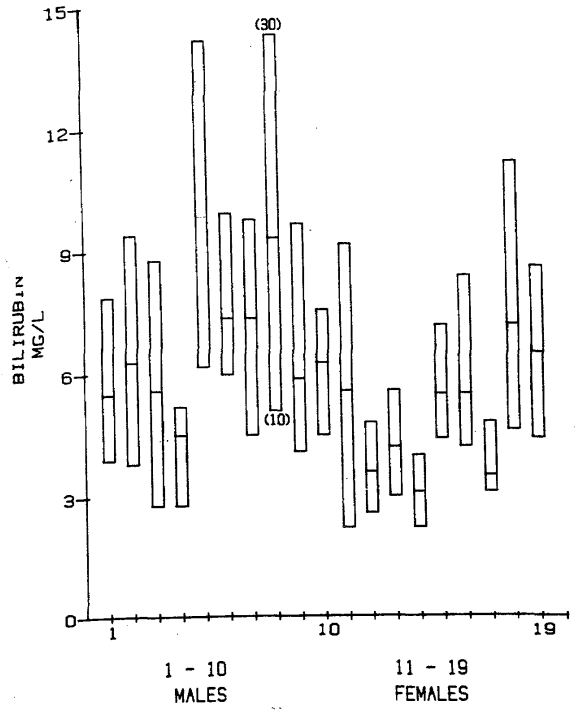

Fig. 5. Intra-individual variation in total bilirubin in serum of 19 apparently healthy individuals

Nos. 1–10, men; 11–19, women. In each bar, midline is the average TBIL concn, top and bottom are highest and lowest observed values. In sample no. 8, the lowest and highest bilirubin values were 10 and 30 mg/L, respectively

candidate Reference Method. Addition of 2 g of hemoglobin per liter to serum samples having BIL concentrations of 13 mg/L and 23 mg/L caused only a 4% decrease in the apparent BIL concentration. Addition of 20 and 50 mg of ascorbic acid per liter to the same samples caused a respective 2% and 5% decrease in the apparent BIL concentration. Both interferences are negligible.

With B_u standards made in human serum, we found the ϵ value of the azopigment to be 1 to 2% higher with this procedure than with the Reference Method. Furthermore, the recovery of B_u added to serum was about 102% when it was calculated with B_u standards in BSA matrix. These findings were ascribable to the different zinc contents of the 40 g/L BSA solution and serum: 7 and 14 μmol/L for the BSA and serum, respectively, or 1.2 and 2.4 μmol/L in the final reaction mixture. The increase in the ϵ value and the percent recovery is explained by the higher zinc concentration of the serum pool (Table 7).

Discussion

The proposed Reference Method for serum TBIL is simple, precise, and almost free of interference from substances that are naturally present in serum or are administered during treatment of disease (*31*).

Standardization

Use of the SRM 916 does not necessarily ensure that standard solutions prepared with this material are accurate. The small probable error of weighing out a rather small amount of bilirubin (about 20 mg) and the greater possible error resulting from incomplete dissolution of the BIL require that the BIL concentration in the standard solution be verified by analysis and calculation of the molar absorptivity, a verification that also serves to ensure that the reagents are of good quality. Bilirubin standard solutions may be prepared by using BIL from other sources, if they are assayed against SRM 916.

The low CVs for the ϵ values of the B_u azopigment, 0.7% for the SRM 916 (this study) and 1.0% obtained in 10 laboratories in the United States (*17*), indicate that BIL standard solutions can indeed be prepared with good reproducibility. Because the ϵ value for the B_u azopigment does not vary with the protein matrix, we recommend the use of BSA Cohn Fraction V for preparing such solutions; it is readily available and less expensive than the corresponding human albumin.

Bilirubin Isomers

Some commercially available BIL preparations, including SRM 916, contain substantial amounts of the III-α and XIII-α isomers of bilirubin (*42*). In contrast, BIL formed in vivo consists exclusively (>99%) of the naturally occurring IX-α isomer (*42*), its isomerization being prevented in serum by the presence of albumin (*43*).

Solutions of the three isomers in $CHCl_3$ exhibit different ϵ values and absorption maxima (*42*): III-α, 65 200 L $mol^{-1}cm^{-1}$ (455–458 nm); IX-α, 62 600 L $mol^{-1}cm^{-1}$ (453–455 nm); XIII-α, 52 500 L $mol^{-1}cm^{-1}$ (449–453 nm). Molar absorptivity values obtained at NBS (by R. G. Christensen) were 62 400, 61 700, and 49 000 L $mol^{-1}cm^{-1}$ for the III-α, IX-α, and XIII-α isomers, respectively.

In the diazo reaction the IX-α isomer yields equimolar amounts of two isomeric azopigments. Each of the symmetrically substituted III-α and XIII-α isomers gives a unique azopigment. Calibration with a standard consisting of a mixture of the three isomers could introduce inaccuracy in the measurement of TBIL in serum if isomers III-α and XIII-α are present in non-equimolar quantities, and if the ϵ values of the two azopigments are not identical. As yet the ϵ values of the two isomeric azopigments have not been directly measured. However, the similar absorptivity values for the SRM 916 and the IX-α isomer (see *Molar Absorptivity*) suggest that if there is a difference, it is small.

Linearity, Precision, and Accuracy

Linearity. Linear regression analysis of the data shown in Figures 1–3 confirm previous observations (*6*) that the relation is linear and that there is adherence to Beer's law up to an absorbance of at least 1.92 *A*.

This not only holds true for B_u but also for pooled sera, which most likely contain all of the BIL species. This statement is based on the small standard errors of the slopes (ranging from 0.3% to 0.9% of the slope values) and on intercepts that are not significantly different from zero (*44*). Pearson's coefficients (r^2) are 1.0 but, in our experience, the use of this statistic is of questionable value in assessing adherence to Beer's law; visual inspection of linear plots may reveal deviations from Beer's law even when the r^2 is 1.0.

Precision. Data on interlaboratory precision have been published elsewhere (*17*). The within-run precision, calculated from duplicate determinations (Table 5), is excellent (CV 0.1%). The intralaboratory between-run CVs (n = 20) for four control sera with TBIL concentrations of 8.1, 57.7, 102.1, and 177.2 mg/L were 3.7, 0.5, 0.7, and 0.7%, respectively. The precision exceeds the criterion recommended by Barnett (*45*), and meets the goals set at the Aspen Conference (*46*), i.e., that the analytical CV should not exceed one-half of the intra-individual CV.

Accuracy. In the absence of definitive methods for all of the BIL species, it is impossible to ascertain that the proposed method measures accurately all of the BIL forms that might be found in serum. The question is, do all of the

BIL species react quantitatively in the proposed method? We know that during the 10-min coupling time (*47*), B_u and dB_c react quantitatively; we know of no reason why mB_c should not also react completely. Furthermore, we know that these three BIL species behave as though they yield the same azopigment (that of B_u) in the reaction mixture (partial hydrolysis would have no effect, for the absorptivities of the B_c and B_u azopigments appear to be identical). Thus, it would be reasonable to conclude from the evidence that B_u, dB_c, and mB_c are measured accurately by the proposed method. However, it is uncertain at this time whether the half of the azopigment formed from B_δ—the one that is firmly bound to protein (*15*)—exhibits the same absorptivity as the B_u azopigment.

The data of Lauff et al. (liquid chromatography vs the Jendrassik–Gróf method) (*16*) suggest that all of the B_δ is measured by the proposed method, but the evidence for complete reaction of B_δ is less conclusive than for the other species. Analyses of a pure B_δ preparation in HSA with the proposed method have shown a linear absorbance–concentration relationship up to 100 mg/L (expressed as B_u), but the yield of azopigment was 10% higher when the diazo reagent concentration was increased by threefold (*48*). The possibility of B_δ being underestimated when the regular-strength diazo reagent is used should be considered with caution, because the reactivity of B_δ in HSA might be different than in whole serum. Data in Table 2 show that values for TBIL in sera from adults, expected to contain B_δ, were somewhat lower (from 2.4 to 3.6%) with the proposed reagent. We believe that, in the measurement of bilirubin in adult sera having high TBIL concentrations (73 to 240 mg/L), such small differences are tolerable because they have no relevance to the diagnosis or monitoring of the disease.

Interferences

Interference from hemoglobin is negligible as long as its concentration does not exceed 2 g/L, a value rarely encountered in sera from neonates (*49*). We recommend adding ascorbic acid (0.1 mL of a 40 g/L solution) before the alkaline tartrate, only if there is gross hemolysis.

The Zn content of serum can introduce small errors in the measured TBIL values. Serum samples having Zn concentrations in the reference range, 10.7 to 22.7 μmol/L (*50*), could contribute to the final reaction mixtures of the two procedures from 0.6 to 1.4 μmol of Zn per liter (volume fraction of sample, 0.059) or from 1.5 to 3.3 μmol/L (volume fraction of sample, 0.17). At these concentrations, Zn causes small but measurable increases in the absorbance of the azopigment (Table 7), which could explain the data in Table 5; i.e., the consistently larger among-sample SDs vs the SDs of duplicates could be due to the differing Zn content of sera and other protein matrices.

Physiological concentrations of ascorbic acid in serum suppress TBIL values negligibly. No significant interference is expected with sera from individuals who are consuming relatively large amounts of vitamin C because, even after ingestion of 1 to 3 g, ascorbic acid concentrations in serum did not exceed 35 mg/L (*51*).

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